

# The *sax1* dwarf mutant of *Arabidopsis thaliana* shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid

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## Summary

Genetic approaches using *Arabidopsis thaliana* aimed at the identification of mutations affecting events involved in auxin signalling have usually led to the isolation of auxin-resistant mutants. From a selection screen specifically developed to isolate auxin-hypersensitive mutants, one mutant line was selected for its increased sensitivity to auxin ( $\times 2$  to  $3$ ) for the root elongation response. The genetic analysis of *sax1* (hypersensitive to abscisic acid and auxin) indicated that the mutant phenotype segregates as a single recessive Mendelian locus, mapping to the lower arm of chromosome 1. *Sax1* seedlings grown *in vitro* showed a short curled primary root and small, round, dark-green cotyledons. In the greenhouse, adult *sax1* plants were characterized by a dwarf phenotype, delayed development and reduced fertility. Further physiological characterization of *sax1* seedlings revealed that the most striking trait was a large increase ( $\times 40$ ) in ABA-sensitivity of root elongation and, to a lesser extent, of ABA-induced stomatal closure; in other respects, hypocotyl elongation was resistant to gibberellins and ethylene. These alterations in hormone sensitivity in *sax1* plants co-segregated with the dwarf phenotype suggesting that processes involved in cell elongation are modified. Treatment of mutant seedlings with an exogenous brassinosteroid partially rescued a wild-type size, suggesting that brassinosteroid biosynthesis might be affected in *sax1* plants. Wild-type sensitivities to ABA, auxin and gibberellins were also restored in *sax1* plants by exogenous

application of brassinosteroid, illustrating the pivotal importance of the BR-related *SAX1* gene.

## Introduction

Cellular, molecular and genetic approaches have led to the cloning of a few genes which play a role in auxin action, but many of the events involved in auxin signalling pathways have still to be identified (Walden and Lubenow, 1996). *Arabidopsis thaliana* mutants affected in auxin responses have been selected on the basis of root phenotypes associated with auxin resistance. They also display alterations in plant morphology and elongation capacity (*axr1*, Estelle and Somerville, 1987; *axr2*, Wilson *et al.*, 1990; *axr3*, Leyser *et al.*, 1996) and/or in gravitropism (*axr2*, Wilson *et al.*, 1990; *aux1*, Timpte *et al.*, 1995). In addition to auxin resistance of root elongation, all these mutations confer cross-resistance to other hormones: ethylene and cytokinins for *axr1* (Timpte *et al.*, 1995), *aux1* (Pickett *et al.*, 1990) and *axr3* (Leyser *et al.*, 1996) or ethylene and abscisic acid for *axr2* (Wilson *et al.*, 1990). These pleiotropic phenotypes of auxin-resistant mutants may illustrate the existence of cross-talk between various hormone signalling pathways. No selection procedure aimed at the screening for auxin hypersensitive mutants has been described thus far. However, the *Arabidopsis* mutant *agr3* (Bell and Maher, 1990) that was initially selected for alterations in root gravitropism also showed an increased auxin sensitivity of root elongation (Maher and Bell, 1990). Auxin hypersensitivity has sometimes been postulated on the basis of morphological traits reminiscent of auxin action on plant development, for instance increased apical dominance in *axr3* (Leyser *et al.*, 1996).

These data emphasize the necessity to search for new mutants exhibiting an increased sensitivity to auxin. Screening for such mutations, aside from broadening the spectrum of auxin response mutants, might allow the identification of negative regulators of auxin sensitivity. The present work describes the isolation of a new *A. thaliana* mutant, *sax1* (hypersensitive to abscisic acid and auxin), originally selected for its increased sensitivity to auxins at the root level. The physiological characterization of *sax1* revealed additional phenotypic traits, including an increased sensitivity to abscisic acid (ABA) of root elongation and stomatal aperture, a resistance of hypocotyl elongation to gibberellic acid (GA<sub>3</sub>) and to ethylene and a

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reduced size. Most of these phenotypic traits could be partially restored by application of exogenous brassinosteroids. Therefore, this mutant provides a new genetic tool for studying interactions between signalling networks involved in hormonal responses of vegetative tissues.

## Results

### *Mutant isolation and genetic mapping*

We screened approximately 500 independent  $M_2$  families for their hypersensitivity to auxin. The screen was based on the search for plantlets exhibiting an exaggerated auxin-induced plant morphology *in vitro*, in the presence of an auxin concentration ( $0.1 \mu\text{M}$  1-naphthaleneacetic acid, NAA), which does not significantly modify the wild-type phenotype (Figure 1a). Seedlings were isolated which exhibited all or some of the phenotypic traits characterizing a wild-type seedling grown on a higher NAA concentration ( $1 \mu\text{M}$ ), i.e. a short and thick primary root, long and dense root hairs and a few secondary roots (Figure 1a). One mutant line was selected as a putative auxin-hypersensitive mutant on these criteria. Because subsequent physiological analysis demonstrated that this mutant is hypersensitive to abscisic acid and to auxin, the mutant was named *sax1*. We did not identify any other allele of *sax1*, either in the EMS  $M_2$  populations tested or in 4000 T2 T-DNA families screened from the T-DNA collection produced by Bechtold *et al.* (1993).

As shown in Figure 1b, the *sax1* mutant phenotype in the absence of auxin is marked enough to be used for performing segregation analyses, its main characteristics being a short primary root, the absence of secondary roots and epinastic cotyledons. The  $F_1$  plants resulting from back-crosses of *sax1* plants to wild-type plants, irrespective of the female parent, all exhibited a wild-type phenotype. In the  $F_2$  progenies, the *sax1* phenotype segregated in a 1:3 ratio, indicating that it is caused by a single recessive Mendelian allele. To map the mutation, homozygous *sax1* plants (Columbia-0 ecotype, Col-0) were crossed to wild-

type plants of the Landsberg ecotype and the  $F_2$  mutant progeny was scored for segregation of CAPS or SSLP markers between the two ecotypes. The *sax1* mutation was located on the lower arm of chromosome 1, between the two markers GAPB and nga 280, at  $15.5 \pm 6.2 \text{ cm}$  and  $11.1 \pm 4.8 \text{ cm}$ , respectively. This region does not contain any published mutation conferring a *Sax1*<sup>-</sup> phenotype (Lister and Dean, 1993), and therefore no allelism tests were carried out between *sax1* and other mutants.

### *The sax1 mutation has pleiotropic effects on plant morphology*

Compared with wild-type seedlings grown for 9 days *in vitro* in the absence of exogenous auxin, *sax1* seedlings grown in the same conditions have much shorter roots and hypocotyls and the cotyledons are dark-green, round, smaller and epinastic (Figure 1b). Moreover, young mutant plants exhibit a strongly reduced production of secondary roots and a root system with longer and denser root hairs. In *sax1* plants, geotropism of root and hypocotyl is not affected. Under dark-growth conditions, *sax1* seedlings display a phenotype closer to that of wild-type and, in particular, cotyledons are etiolated, the root has almost a normal length, and although the hypocotyl is still 50% shorter than the wild-type, it displays an approximately 10-fold increase in length in response to darkness as seen in the wild-type (Figure 1b).

When grown in the greenhouse, mutant plants show a strongly altered phenotype. Their rosette is small and composed of round leaves with short epinastic petioles (Figure 1c, Table 1). Adult *sax1* plants show a very short primary stem as well as shorter secondary branches due to strongly reduced internode length (more than 10 times, see Table 1), which account for the dwarf stature of *sax1* plants (Figure 1c). In flowering plants, *sax1* shows fewer inflorescences and siliques, and fertility is reduced (Table 1). The time-course of development up to the adult mutant plant is delayed by 4–5 days for most of the developmental events, including the occurrence of the first

**Figure 1.** Physiological basis of the screen and comparison of the morphology of wild-type and *sax1* mutant plants. (a) Auxin effects on the morphology of wild-type seedlings grown for 7 days *in vitro* on 0,  $0.1 \mu\text{M}$  and  $1 \mu\text{M}$  NAA (from left to right). Bar = 0.55 cm. (b) Morphology of seedlings grown for 9 days *in vitro* in the light (16 h light/8 h dark) or in the dark. In each condition, seedlings are shown for wild-type (left) and mutant (right) genotypes. Bar = 0.45 cm. (c) Seven-week-old wild-type (left) and mutant (right) plants grown in greenhouse conditions.

**Figure 5.** Effects of 24-epibrassinolide on growth of wild-type and *sax1* mutant seedlings under different light conditions. (a) Effect of the brassinosteroid on *sax1* morphology *in vitro*. Wild-type and mutant seedlings were grown for 8 days in the absence (–) or presence of  $10^{-9} \text{ M}$  24-epibrassinolide (+). Bar = 0.55 cm. (b,c) Seedlings were grown *in vitro* for 7 days under light (16 h light/8 h dark cycle) on medium supplemented with different EBR concentrations. Data points represent the mean of 30 measurements  $\pm$  SE. Dose–response curves of root growth (b) and hypocotyl growth (c) are shown for one representative experiment out of three independent experiments. (d,e) Seedlings were grown *in vitro* for 7 days in darkness on medium supplemented with different EBR concentrations. Data points represent the mean of 30 measurements  $\pm$  SE. Dose–response curves of root growth (d) and hypocotyl growth (e) are shown for one representative experiment out of three independent experiments.

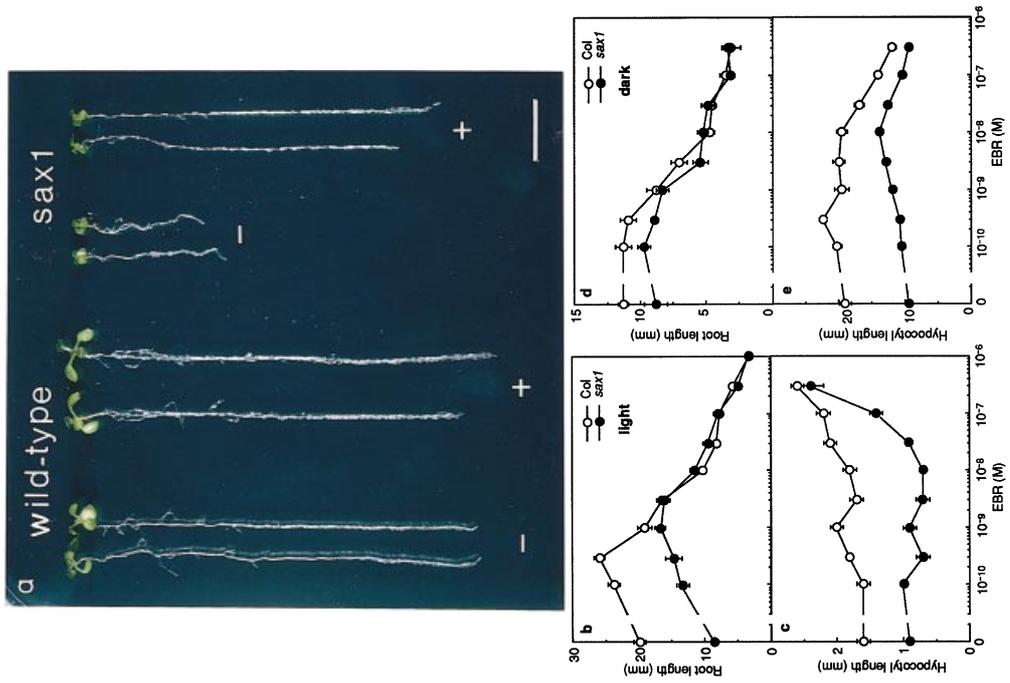


Figure 5.

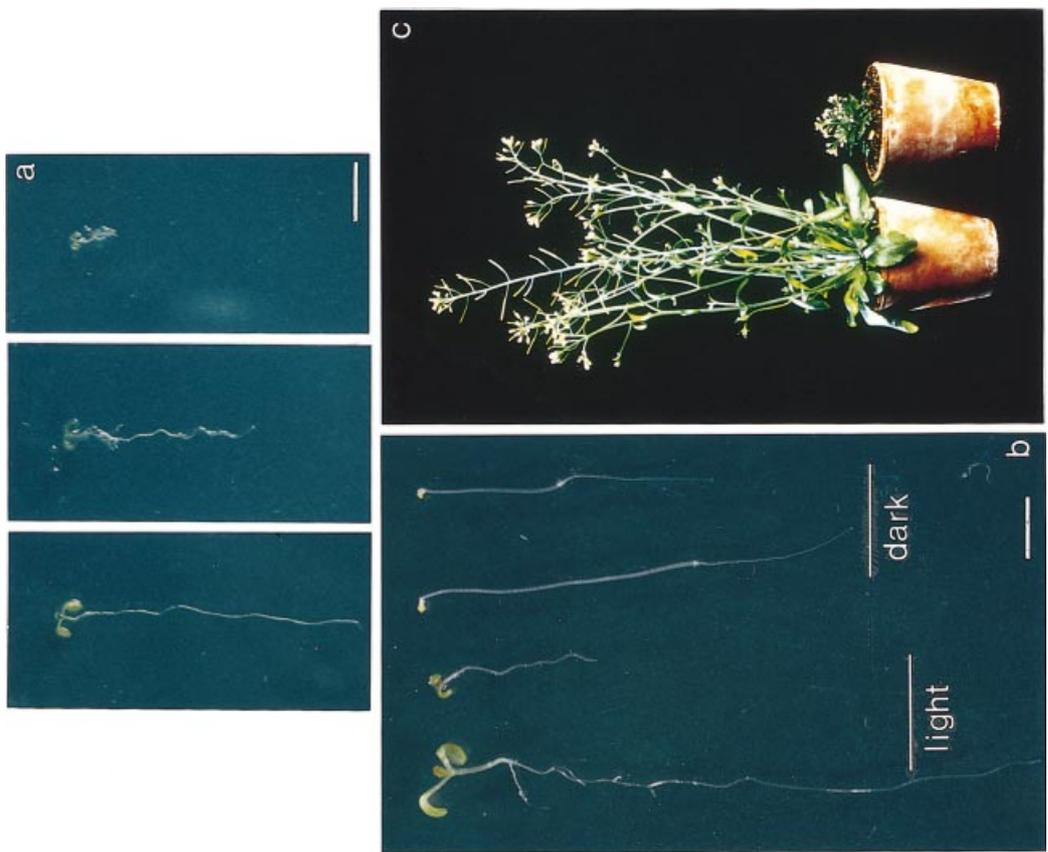


Figure 1.

**Table 1.** Morphometric characteristics of wild-type and *sax1* plants grown in the greenhouse

	Sax1 <sup>+</sup>	Sax1 <sup>-</sup>
Rosette diameter (cm)	15.0 ± 0.4	4.5 ± 0.1
Height of primary stem (cm)	35.5 ± 0.7	3.6 ± 0.1
Number of side branches	3.6 ± 0.4	3.7 ± 0.3
Number of secondary branches	7.5 ± 0.3	3.4 ± 0.3
Number of inflorescences	47 ± 3	14 ± 5
Internode distance (cm) (first and second ones)	3.1 ± 0.4	0.2 ± 0.02
Number of siliques/plant	220 ± 19	36 ± 4
Approximate number of seeds per silique after 8 weeks	66 ± 8	35 ± 5

Seedlings were germinated and grown in the greenhouse for 6 weeks in a 16 h light/8 h dark cycle. Data represent mean ± standard error of 10 plants.

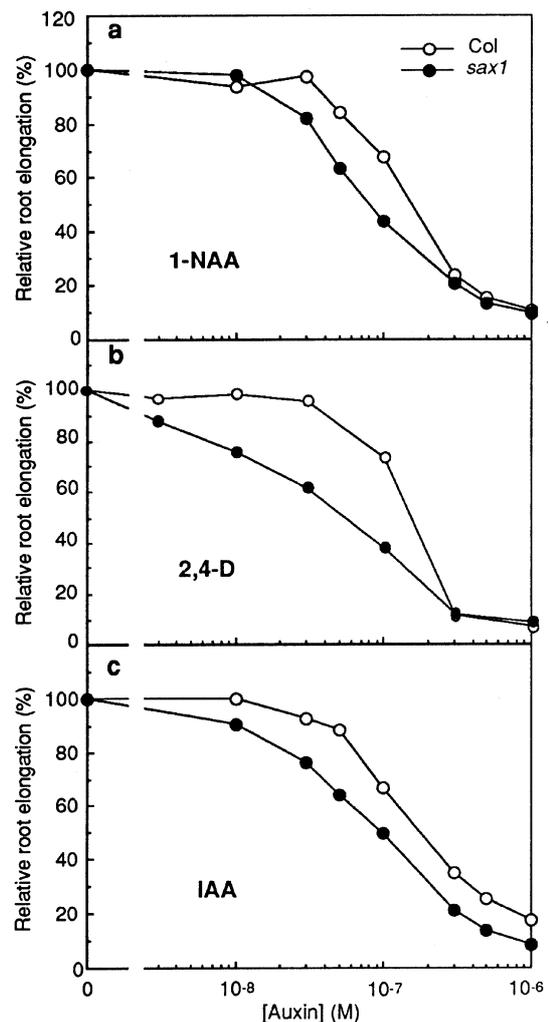
side branch, secondary branches and inflorescence and opening of the first flower. The largest delay (1 week) was observed for the senescence of flowers and siliques.

#### Root elongation in *sax1* seedlings is hypersensitive to auxin

Root growth inhibition by auxins was analysed in wild-type and *sax1* genotypes. The results of the different assays are expressed as a percentage of growth in the absence of hormone, allowing direct comparisons of the two genotypes despite the short root of *sax1* and its slow rate of elongation. Routinely, the seeds were germinated directly on 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) and root length was scored after 7 days (Figure 2). For both genotypes, increasing auxin concentrations in the culture medium inhibited root growth up to 80–90% in the presence of 1 µM auxin. However, determination of the concentrations inducing a 50% inhibition indicate that *sax1* roots are more sensitive to auxins by a factor ranging from 2 (for NAA and IAA) to 3 (for 2,4-D). In contrast, the roots of *sax1* show wild-type sensitivities to two other hormones which inhibit root elongation, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the cytokinins kinetin and benzyladenine (data not shown).

#### Root elongation and stomatal closure are hypersensitive to ABA in *sax1* plants

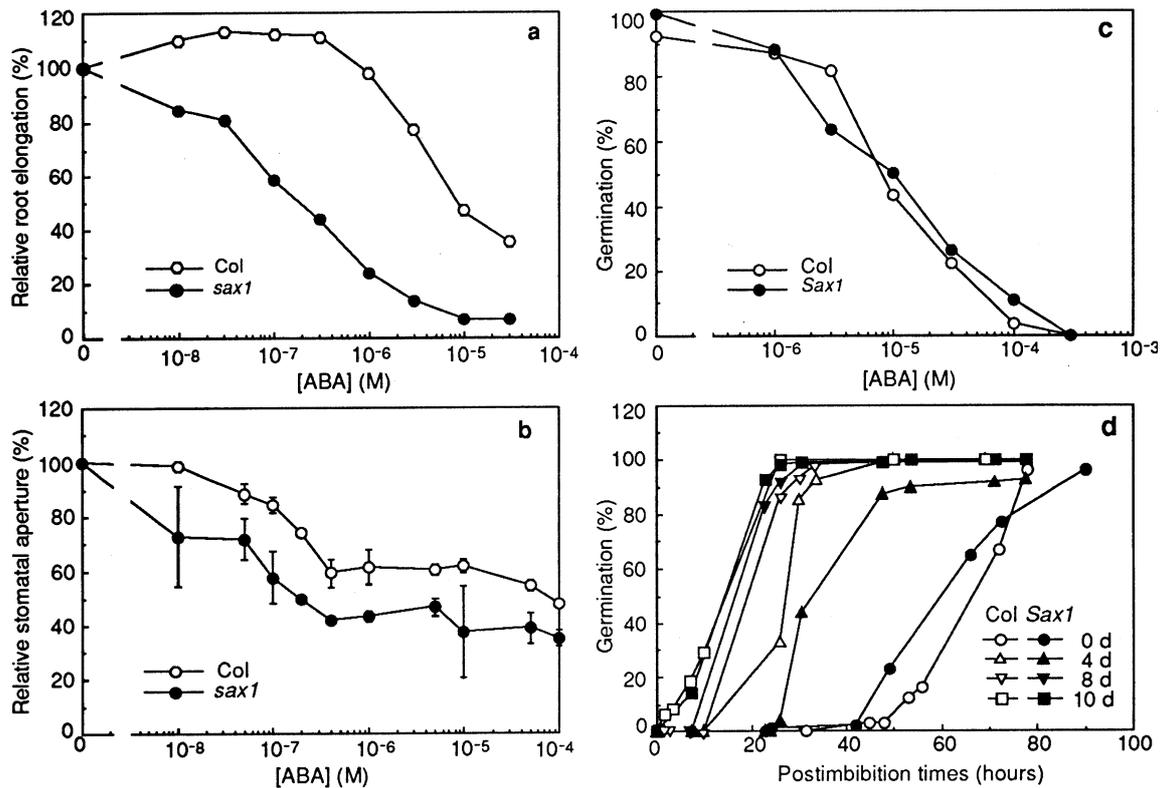
The increased sensitivity of root elongation in *sax1* is not specific to auxin. The *sax1* mutation also confers an increased sensitivity to abscisic acid, as root growth was inhibited by ABA concentrations 40 times lower than those effective on wild-type roots (Figure 3a). This hypersensitivity to ABA co-segregated with both the hypersensitivity

**Figure 2.** Effects of auxins on the root elongation of wild-type and *sax1* mutant seedlings.

Each value represents the mean of at least 30 measurements on 7-day-old seedlings grown on NAA (a), 2,4-D (b) and IAA (c). Inhibition of root growth is expressed relative to the mean growth of the same genotype on medium without hormones. The standard errors on percentage inhibition are not represented because they never exceed 6%. Mean values for 100% root growth were: (a) wild-type 22.2 ± 0.7 mm, *sax1* 10.4 ± 0.4 mm; (b) wild-type 24.3 ± 0.6 mm, *sax1* 10.9 ± 0.4 mm; (c) wild-type 27.0 ± 0.5 mm, *sax1* 11.3 ± 0.4 mm. Dose-response curves are shown for one representative experiment out of at least two independent experiments.

to auxins and the morphological phenotype in F<sub>3</sub> mutant seedlings raised from the two successive back-crosses.

To test the ABA-induced closure of stomata, epidermal strips were incubated in the light for 2 h to fully open the stomata before adding ABA. The relative stomatal aperture is reported in Figure 3b for the two genotypes as a function of ABA concentrations. Increasing concentrations of ABA decreased the aperture of wild-type and *sax1* stomata. Although *sax1* stomata displayed a higher variability in this response than stomata from wild-type plants, they appear more sensitive to ABA (Figure 3b). The stomatal density in the abaxial epidermis does not differ significantly



**Figure 3.** ABA responses in wild-type and *sax1* mutant plants.

(a) Effect of ABA on root elongation. Each value represents the mean of at least 30 measurements on 7-day-old seedlings grown for the last 2 days (from days 5–7) on ABA after transfer. Inhibition of root growth is expressed relative to the mean growth of the same genotype on medium without hormones. The standard errors on percentage inhibition are not represented because they never exceed 6%. Mean values for 100% root growth were: wild-type  $24.0 \pm 0.4$  mm, *sax1*  $8.8 \pm 0.2$  mm. Dose–response curves are shown for one representative experiment out of three independent experiments.

(b) Effect of ABA on the closure of wild-type and *sax1* mutant stomata. Epidermal strips were floated under light ( $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h on 60 mM KCl, 10 mM MES, pH 6.15, then for a further 3 h in the same medium supplemented with a range of ABA concentrations. Measurements were performed on 100 stomata. As regards the variability in the response of *sax1* stomata, mean values and the corresponding standard errors have been calculated from between two and eight independent experiments, depending on the ABA concentration tested. Mean values of aperture width before adding ABA (taken as 100%) were: wild-type  $5.13 \pm 0.21 \mu\text{m}$ , *sax1*  $3.49 \pm 0.15 \mu\text{m}$ .

(c) Effect of ABA on seed germination. Germination was scored as positive when a radicle tip had fully penetrated the seed coat. Data from one experiment are shown. Similar results were obtained in three independent experiments.

(d) Seed dormancy. Mature seeds were chilled for periods ranging from 0 to 10 days at 4°C in darkness. Germination was scored as described in (c). Data from one experiment are shown. Similar results were obtained in two independent experiments.

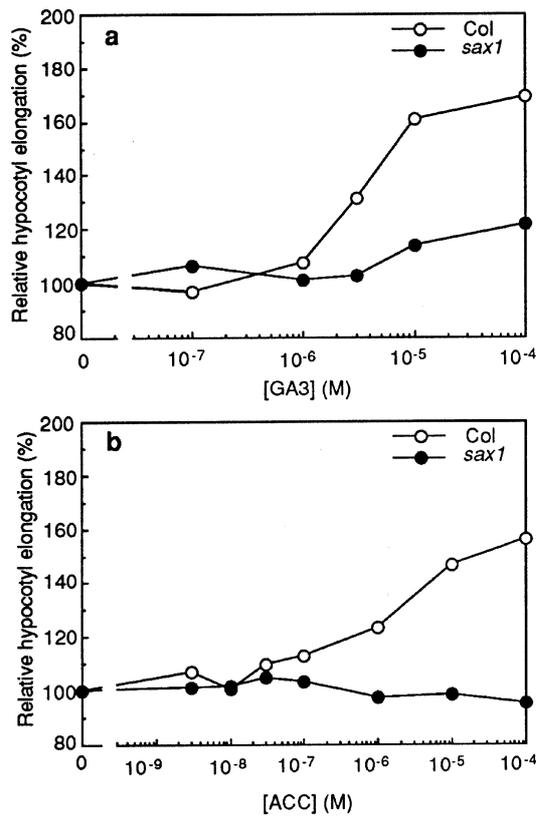
between wild-type ( $297 \pm 16 \text{ mm}^{-2}$ ,  $n = 8$  independent estimations) and *sax1* leaves ( $326 \pm 38 \text{ mm}^{-2}$ ,  $n = 8$  independent estimations). The mean length of guard cells is identical for wild-type and mutant stomata,  $18.8 \pm 0.4 \mu\text{m}$  ( $n = 2$  independent experiments,  $2 \times 100$  cells) and  $17.6 \pm 0.3 \mu\text{m}$  ( $n = 2$  independent experiments,  $2 \times 100$  cells), respectively. However, the stomatal pore width in the mutant is smaller than in the wild-type plants, both in the dark ( $0.94 \pm 0.07 \mu\text{m}$  and  $1.91 \pm 0.16 \mu\text{m}$ , respectively,  $n = 5$  independent experiments,  $5 \times 100$  pores), as well as in the light ( $3.49 \pm 0.15 \mu\text{m}$  and  $5.13 \pm 0.21 \mu\text{m}$ , respectively,  $n = 11$  independent experiments,  $11 \times 100$  pores). Thus, these properties cannot account for the ABA-hypersensitivity of the *sax1* stomata.

In other respects, seed responses are not altered in *sax1* plants. Germination of dry seeds from wild-type and *sax1* plants exhibited the same sensitivity to exogenous ABA,

with 50% inhibition occurring in the presence of  $10 \mu\text{M}$  ABA (Figure 3c). The efficiency of germination of seeds from wild-type and *sax1* plants is identical under post-imbibition conditions without cold treatment (50% germination after 60 h post-imbibition) and increases almost proportionately to the length of the chilling period (Figure 3d). Furthermore, the ABA-induced expression of the *Rab18* and *AtDi21* genes (Gosti *et al.*, 1995) is not modified in *sax1* plants (data not shown).

#### *Hypocotyl elongation of sax1 seedlings in the light is insensitive to gibberellins and ethylene*

On light-grown seedlings, the elongation of wild-type hypocotyls was promoted by increasing concentrations of  $\text{GA}_3$  while the hypocotyl of *sax1* plants was resistant to the hormone and almost did not elongate, with the highest  $\text{GA}_3$



**Figure 4.** Effects of GA<sub>3</sub> and ACC on hypocotyl elongation of wild-type and *sax1* mutant seedlings.

Each value represents the mean of at least 30 measurements on 7-day old seedlings grown on GA<sub>3</sub> (a) or ACC (b). Stimulation of hypocotyl growth is expressed relative to the mean growth of the same genotype on medium without hormones. The standard errors on percentage stimulation are not represented because they never exceed 6%. Mean values for 100% hypocotyl growth were: (a) wild-type  $1.9 \pm 0.1$  mm, *sax1*  $0.80 \pm 0.04$  mm; (b) wild-type  $2.2 \pm 0.1$  mm, *sax1*  $0.90 \pm 0.04$  mm. Dose-response curves are shown for one representative experiment out of at least two independent experiments.

concentration tested ( $10^{-4}$  M) stimulating the elongation by only 20% (Figure 4a). The dwarf phenotype of *sax1* plants grown in the greenhouse could not be reversed by gibberellin treatments (1 mM GA<sub>3</sub>) although the treated plants displayed a slight increase of main stem growth and fertility, two responses characterizing GA<sub>3</sub> action (data not shown).

When ACC (1-aminocyclopropane-1-carboxylic acid) was tested in light conditions (16 h light/8 h dark cycle), it stimulated hypocotyl elongation of wild-type plants when applied at concentrations higher than  $10^{-7}$  M (Figure 4b). This result, in agreement with data reported recently by Smalle *et al.* (1997), was highly reproducible and was also obtained with ethylene (A. Barry and M. Hall, personal communication). In contrast, hypocotyl elongation of *sax1* plants was affected neither by ACC, whatever the concentration tested (Figure 4b), nor by ethylene (data not shown), although *sax1* efficiently converts ACC in ethylene (A.

Barry and M. Hall, personal communication). These results indicate that hypocotyls of *sax1* plants are insensitive to ethylene under light conditions although a normal triple response to ACC in dark conditions is observed (data not shown).

#### *The dwarf phenotype of sax1 seedlings is rescued by 24-epibrassinolide*

Brassinosteroids have been shown to play an essential role in plant development, especially in the control of cell elongation (reviewed in Clouse, 1996). Wild-type and mutant plants were treated by the brassinosteroid 24-epibrassinolide (EBR). As shown in Figure 5a, the short phenotype of *sax1* mutant plants grown in the light is partially rescued by addition of  $10^{-9}$  M EBR. The more obvious trait is the long root of *sax1* seedlings after an EBR treatment, the aerial part not being significantly modified in these conditions.

A wide range of 24-epibrassinolide (EBR) concentrations were tested under light conditions on root and hypocotyl elongation of wild-type and mutant genotypes. The dose-response curves show that root elongation in wild-type plants was stimulated by at most 20% in the presence of  $3 \times 10^{-10}$  M EBR and higher concentrations are inhibitory (Figure 5b). Application to *sax1* seedlings of EBR concentrations lower than  $10^{-9}$  M induced a large increase in root length, reaching a maximal stimulation of 100% at  $10^{-9}$  and  $3 \times 10^{-9}$  M, whereas higher EBR concentrations inhibit root elongation (Figure 5b). EBR ( $3 \times 10^{-7}$  M) also stimulated hypocotyl elongation of wild-type and mutant seedlings up to 100% and 200%, respectively (Figure 5c), leading to a complete restoration of hypocotyl length in *sax1* plants. In both genotypes, the hypocotyl is less sensitive to EBR than the root by two orders of magnitude. Table 2 shows morphometric characteristics of root and hypocotyl of *in vitro* wild-type and *sax1* plants cultivated in the light, in the absence or presence of EBR concentrations that were the most efficient on roots ( $3 \times 10^{-9}$  M) and hypocotyls ( $3 \times 10^{-7}$  M) (Figure 5b,c). Mutant primary roots are approximately 60% shorter than those of the wild-type, whereas their epidermal cell lengths are identical (Table 2) which suggests that mutant roots have a reduced number of cells. The addition of  $3 \times 10^{-9}$  M EBR in the medium has no significant effect on wild-type seedlings whereas a large increase in *sax1* root length is observed, with the size of epidermal cells being unchanged in the mutant root (Table 2). In the case of hypocotyls, the epidermal cells are half the length of wild-type, which fits well with the reduced hypocotyl length. For the two genotypes, the addition of  $3 \times 10^{-7}$  M EBR in the medium increases hypocotyl and cell lengths. Under those conditions, the values reached in *sax1* are identical to those of wild-type plants (Table 2).

Under dark-growth conditions, EBR concentrations

**Table 2.** Morphometric characteristics of wild-type and *sax1* plants grown *in vitro* in the absence (control) or presence of 24-epibrassinolide (+ EBR)

		ROOT			
		Length (mm)		Epidermal cell length ( $\mu\text{m}$ )	
		control (+ $3 \times 10^{-9}$ M EBR)		control (+ $3 \times 10^{-9}$ M EBR)	
WT		21.3 $\pm$ 4.8	22.7 $\pm$ 2.3	98.7 $\pm$ 6.5	94.9 $\pm$ 5.1
<i>sax1</i>		8.7 $\pm$ 0.2	16.7 $\pm$ 0.4	96.7 $\pm$ 2.5	96.7 $\pm$ 8.9
		HYPOCOTYL			
		Length (mm)		Epidermal cell length ( $\mu\text{m}$ )	
		control (+ $3 \times 10^{-7}$ M EBR)		control (+ $3 \times 10^{-7}$ M EBR)	
WT		1.9 $\pm$ 0.3	3.0 $\pm$ 0.7	82.4 $\pm$ 5.1	124.4 $\pm$ 19.1
<i>sax1</i>		0.9 $\pm$ 0.3	2.2 $\pm$ 0.5	63.6 $\pm$ 7.6	135.1 $\pm$ 18.1

Seedlings were germinated and grown on ABIS medium for 7 days in a 16 h light/8 h dark cycle. Data represent mean ( $\pm$  SE) of three plants (for organ measurement,  $n = 3$ ) and 30–40 cells from each of the three plants (for cell measurements,  $n = 90$ –120). A second independent experiment performed in the same conditions gave similar results.

ranging from  $10^{-9}$  to  $3 \times 10^{-7}$  M display an increasing inhibition of root elongation for both genotypes (Figure 5d). Hypocotyl elongation in *sax1* is significantly stimulated by  $10^{-8}$  M EBR, but higher concentrations inhibit hypocotyl growth in both genotypes (Figure 5e).

*Treatment with EBR abolishes the hypersensitivity of sax1 root elongation to ABA and auxin and restores the capacity of the sax1 hypocotyl to respond to GA but not to ACC*

Apart from its dwarf stature, *sax1* displays alterations in the responsiveness to hormonal regulation of cell elongation, both in the root and in the hypocotyl. The effects of EBR treatment on these phenotypic traits were evaluated. As to the root elongation, the addition of  $10^{-9}$  M EBR, the most efficient concentration to promote root elongation, to the media supplemented with different ABA (Figure 6a) or IAA (Figure 6b) concentrations does not affect the dose-response curves of wild-type roots but totally suppresses the shifts in sensitivity to ABA or auxin observed in *sax1* root.

The *sax1* hypocotyl length was unaffected by  $\text{GA}_3$  and ACC treatments (Figure 6c,d, respectively). When  $3 \times 10^{-7}$  M EBR, the most efficient concentration on hypocotyl, was added to increasing  $\text{GA}_3$  concentrations in the culture medium, both genotypes displayed a high stimulation of hypocotyl elongation in wild-type plants and also clearly in *sax1* plants (Figure 6c). This indicates that *sax1* hypocotyl has recovered the capacity to respond to gibberellin in a dose-dependent manner in the presence of EBR. The addition of EBR ( $3 \times 10^{-7}$  M) to ACC-containing media inhibits

ethylene stimulatory action in wild-type seedlings and therefore does not reveal a responsiveness of hypocotyl elongation in *sax1* seedlings (Figure 6d).

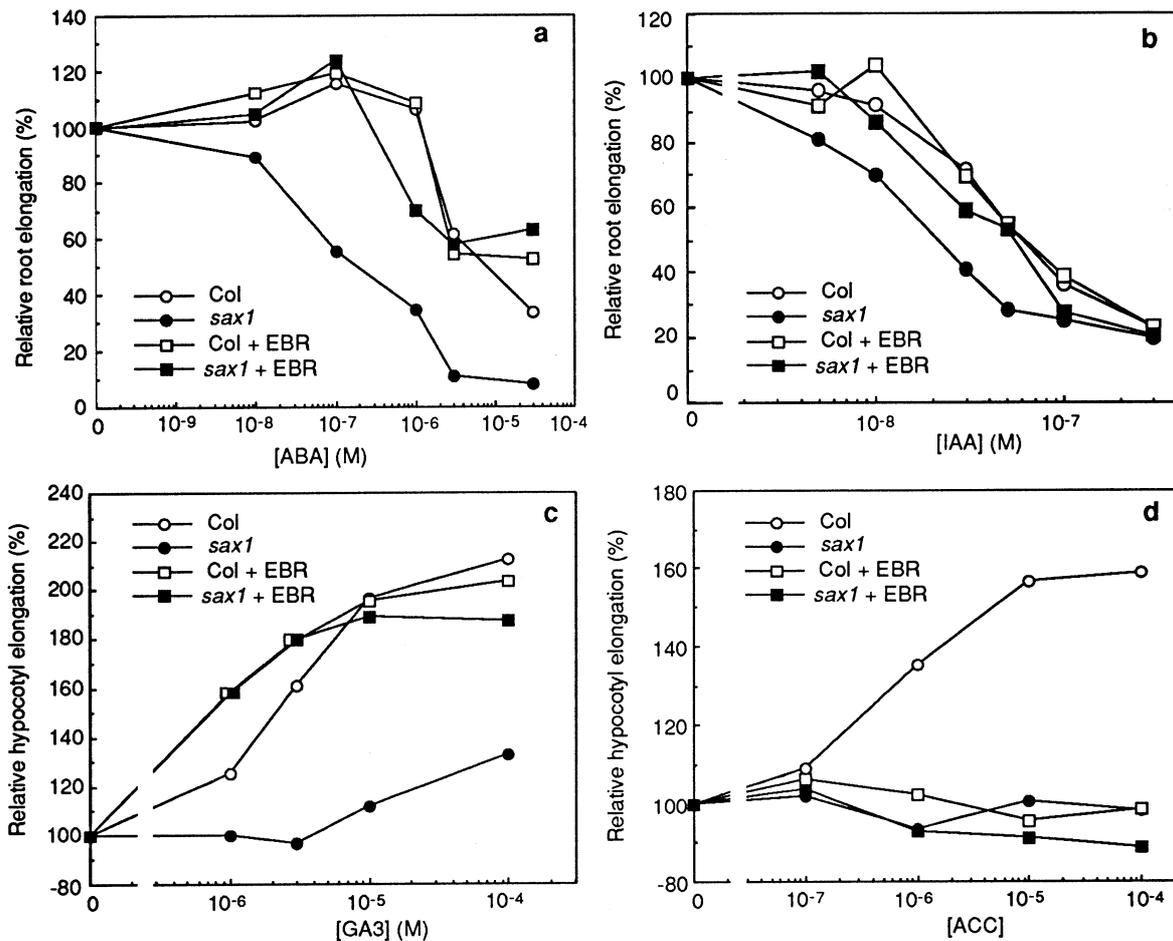
## Discussion

We have isolated a new recessive nuclear mutation, *sax1*, which maps to a single locus on chromosome 1. The *sax1* mutation has pleiotropic effects on the morphology of seedlings grown *in vitro*, as well as of plants in the greenhouse, the more striking trait being pronounced dwarfism. The *sax1* mutant is also characterized by an increased sensitivity of root elongation to abscisic acid and, to a lesser extent, to auxin and by a resistance of hypocotyl growth to gibberellins and to ethylene in light conditions. All these phenotypic traits co-segregate which suggests that they originate from a single mutation.

Several dwarf mutants affected either in gibberellin biosynthesis or in response to gibberellins have been described previously (Koornneef and van der Veen, 1980; Koornneef *et al.*, 1985). On the basis of the mapping, *sax1* lies at a different locus. The defect in hypocotyl elongation exhibited by *sax1* seedlings grown *in vitro* in the presence of  $\text{GA}_3$  would probably be due to a lack of responsiveness to gibberellins restricted to hypocotyl as *sax1* plants in the greenhouse showed a partial response to sprayed  $\text{GA}_3$ . The hypocotyl of *sax1* seedlings is also affected in its sensitivity to ethylene, but only in light-growth conditions as *sax1* is normally responsive to ethylene in the dark. These results suggest that the *sax1* mutation does not lead to a general insensitivity to these growth regulators.

Interestingly, with regard to previously described auxin response mutants, *sax1* seedlings show hypersensitivity to auxins, with root elongation being inhibited by auxin concentrations 2–3 times lower than those affecting wild-type roots. This is probably due to a shift in auxin sensitivity, as preliminary results showed no significant difference between *sax1* and wild-type plants in the auxin endogenous content (R. Maldiney, personal communication) or in the accumulation and metabolism of exogenous auxin (data not shown). Up until now, only two *A. thaliana* mutants have been postulated as auxin hypersensitive, *agr3* (Maher and Bell, 1990) and *axr3* (Leyser *et al.*, 1996), but the possibility that the mutations affect a common locus can be ruled out as the corresponding genes are mapped either on chromosome 1 but far from *sax1* in the case of *axr3* (Leyser *et al.*, 1996) or on chromosome 5 for *agr3* (Sinclair *et al.*, 1996).

The *sax1* mutant displays a highly significant increase in ABA sensitivity for several vegetative responses. From preliminary assays for endogenous ABA content, no significant difference was found between *sax1* and wild-type plants (R. Maldiney, personal communication), suggesting that ABA signalling pathways rather than biosynthesis are



**Figure 6.** Effect of 24-epibrassinolide on ABA- or IAA-sensitivity of root elongation and on  $GA_3$ - or ACC-sensitivity of hypocotyl elongation in wild-type and *sax1*-mutant seedlings.

Dose-response curves are shown for one representative experiment out of two (a) and three independent experiments (b, c, d). The standard errors on % inhibition are not shown since they never exceed 6%.

(a, b) Inhibition of root elongation by ABA (a) or IAA (b) is expressed relative to the mean growth of the same genotype on medium without ABA/IAA or with  $10^{-9}$  M EBR. Each value represents the mean of at least 30 measurements on 7-day-old seedlings (see Experimental procedures). Mean values for 100% root elongation were on standard medium: (a) wild-type,  $12.3 \pm 0.3$  mm; *sax1*,  $4.3 \pm 0.2$  mm; on EBR medium: wild-type,  $11.3 \pm 0.2$  mm; *sax1*,  $7.4 \pm 0.4$  mm; (b) wild-type,  $10.7 \pm 0.6$  mm; *sax1*,  $3.1 \pm 0.2$  mm; on EBR medium: wild-type,  $10.9 \pm 0.6$  mm; *sax1*,  $6.3 \pm 0.4$  mm.

(c, d) Stimulation of hypocotyl elongation by  $GA_3$  (c) or ACC (d) is expressed relative to the mean growth of the same genotype on medium without  $GA_3$ /ACC or with  $10^{-9}$  M EBR. Each value represents the mean of at least 30 measurements on 7-day-old seedlings (see Experimental procedures). Mean values for 100% hypocotyl elongation were on standard medium: (c) wild-type,  $1.6 \pm 0.1$  mm; *sax1*,  $1.1 \pm 0.1$  mm; on EBR medium: wild-type,  $4.8 \pm 0.1$  mm; *sax1*,  $4.5 \pm 0.1$  mm; (d) wild-type,  $1.8 \pm 0.1$  mm; *sax1*,  $1.1 \pm 0.1$  mm; on EBR medium: wild-type,  $4.6 \pm 0.1$  mm; *sax1*,  $4.2 \pm 0.2$  mm.

altered by the *sax1* mutation. Up until now, the *era* mutants are the only ones selected in *A. thaliana* as being hypersensitive to ABA (Cutler *et al.*, 1996). The *era1* mutation has not been mapped, but the corresponding gene has been cloned. *ERA1* encodes the  $\beta$ -subunit of a protein farnesyl transferase (Cutler *et al.*, 1996). The recessive nature of the mutation suggests that *ERA1* could act as a negative regulator of ABA signals. Except for an increase in seed dormancy, the physiological characterisation of the *era1* mutant is rather limited. However, it seems that the morphologies of *sax1* and *era1* mutants are distinct in seedlings grown *in vitro* and in the adult plant in the greenhouse, as the authors mentioned that *era1* plants exhibit relatively normal growth and development (Cutler

*et al.*, 1996). In addition, *sax1* in contrast to *era1* does not exhibit an increased seed dormancy, making it unlikely that the *SAX1* and *ERA1* genes are identical. However, a genetic complementation test is needed to rule out the two mutations being allelic. The fact that the *sax1* mutation affects ABA responses characterizing vegetative tissues, namely root elongation and stomatal closure, raises the possibility that *sax1* could be affected in the same pathway as *abi1* and/or *abi2* mutants (reviewed in Merlot and Giraudat, 1997). However, the ABA responses involved in germination, dormancy and in *Rab18* and *AtDi21* gene transcription are not affected by the *sax1* mutation whereas they are strongly altered in *abi1* and *abi2* mutants (Gosti *et al.*, 1995; Koornneef *et al.*, 1984; Leung *et al.*, 1997;

Roelfsema and Prins, 1995). This suggests either that SAX1 lies in an ABA signalling pathway distinct from ABI1 and ABI2 cascades or, more likely, that *sax1* is not primarily defective in an ABA-response component within the primary ABA transduction cascade.

The hypocotyl and root are severely shortened in *sax1* plants compared to wild-type ones, thus accounting for the dwarf phenotype. This dwarf phenotype can be rescued by growing *sax1* plants on EBR, suggesting that *sax1* could be defective in brassinosteroid biosynthesis. The treatment of *sax1* seedlings growing in light conditions by increasing exogenous EBR concentrations partially restores root elongation (100% stimulation) and totally rescues hypocotyl elongation (200% stimulation). Further characterization at the histological level revealed that the *sax1* mutant has epidermal cells which are only half the length of those of wild-type due to defects in hypocotyl elongation. In the case of the retarded root elongation, reduction of cell division has to be postulated as the cause as epidermal cells have a normal length. Consequently, application of exogenous brassinosteroids to *sax1* plants has two different effects – activation of cell division in the root and stimulation of cell elongation in the hypocotyl. The promotion of cell division has been observed in the upper part of brassinosteroid-treated internodes of bean plants (Worley and Mitchell, 1971) and was described recently in isolated leaf protoplasts of *Petunia hybrida* (Clouse, 1998), although such an effect does not seem to be general to all plants. For example, brassinosteroid failed to activate cell division in cultured carrot cells (Sala and Sala, 1985). The efficiency of EBR application on seedlings growing in dark conditions is low; however, the elongation of *sax1* hypocotyl can be increased up to 50% by EBR with no further increase by the natural brassinolide (data not shown). This may result from the fact that the elongation response of *sax1* plants is not strongly altered in the dark as etiolated hypocotyls elongate in the same proportion ( $\times 10$ ) in the mutant and wild-type plants compared to light-grown seedlings. Studies on dwarf mutants of *A. thaliana* have shed light on the role of brassinosteroids in the control of elongation processes (Clouse, 1996; Ecker, 1997). Strong similarities in terms of morphology exist between *sax1* plants and a series of mutants affected in biosynthesis or signalling of brassinosteroids (reviewed in Altmann, 1998). The *det2* locus is located on chromosome 2 (Li *et al.*, 1996), *cpd* mutation on chromosome 5 (Szekeres *et al.*, 1996), the locus of the three alleles, *cbb1*, *dim* and *dwf1*, maps on chromosome 3 (Kauschmann *et al.*, 1996) and the mutation *bri1* on chromosome 4 (Clouse *et al.*, 1996). The *dwf4* mutation was mapped very recently on chromosome 3 (Azpiroz *et al.*, 1998). The location of the *sax1* mutation on chromosome 1 clearly eliminates the possibility that *sax1* is allelic to one of the known brassinosteroid mutants.

Other remarkable characteristics of *sax1* plants are the hypersensitivity of roots to ABA and auxin and the insensitivity of hypocotyls to GA<sub>3</sub> and ACC. These phenotypic traits co-segregate with an abnormal growth development of seedlings grown *in vitro*, as well as in adult plants in the greenhouse. Except for ACC insensitivity, all the hormonal phenotypes were reversed by EBR addition to the growth medium which shifted the dose–response curves to a wild-type sensitivity to IAA and ABA for root elongation and to GA<sub>3</sub> for hypocotyl growth. Clearly, the addition of brassinosteroid to ACC-media does not restore a wild-type sensitivity to ethylene in *sax1* seedlings, and abolishes the response of wild-type seedlings to ACC in the light. This effect on wild-type plants, which reveals the existence of interactions between brassinosteroid and ethylene response pathways in the light, has not been investigated further. These data demonstrate that the primary defect in *sax1* concerns the synthesis of brassinosteroids, and that most of the other phenotypic traits derive from this primary alteration. Very little is known about the sensitivity of brassinosteroid mutants to other hormones. The *bri1* mutant, a mutant affected in brassinosteroid perception, shows an increased response to ABA compared to wild-type plants (Clouse *et al.*, 1996). Among the brassinosteroid biosynthesis mutants, Kauschmann *et al.* (1996) reported that the *cbb1* and *cbb3* mutants react as wild-type seedlings to auxin, cytokinin, gibberellins and an ethylene releasing compound (ethrel). Few data are available concerning the interactions of brassinosteroids with other hormones, as reviewed by Mandava (1988). Basically, in many bioassays, brassinosteroids interact strongly with auxins (possibly synergistically). The responses to brassinosteroids and GA appear to be independent and additive, whereas ABA interacts strongly with brassinosteroids and reverses their growth effects. Recently, from the analysis of the *dwf4* phenotype (Azpiroz *et al.*, 1998) indirect evidence has been presented that a fully active brassinosteroid pathway is necessary for a full response to GA, auxin and darkness.

We provide direct evidence here for the existence of interactions between brassinosteroids and auxin, ABA, GA and ethylene signalling pathways. The fact that brassinosteroids restore a wild-type sensitivity at least to auxin, ABA and GA<sub>3</sub> in *sax1* seedlings strongly demonstrates that three different signalling pathways controlling cell elongation are under the control of a fourth pathway involving brassinosteroids. The absence of GA response in cells defective for elongation might just mean that brassinosteroid is the main limiting factor of elongation and that GA cannot compensate for this. On the contrary, hypersensitivity to ABA and auxin is convincing evidence that brassinosteroid must at some point antagonize ABA and auxin responses. In that case, the target of the brassinosteroids might be a common signalling component to ABA and auxin pathways, or will lie downstream of the signalling

pathways in a step involved in the elongation process *per se*. The *sax1* mutation represents an original tool for identifying common targets to hormone signalling pathways involved in the control of cell elongation.

## Experimental procedures

### Plant material and growth conditions

Seeds from *Arabidopsis thaliana* (L.) Heyn, Columbia-0 ecotype (Col-0) were surface sterilized for 1 h in sodium hypochlorite (0.9% active Cl), rinsed six times with sterile water and stored overnight at 4°C. They were then plated on a medium containing 5 mM KNO<sub>3</sub>, 2.5 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6, 2 mM MgSO<sub>4</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM MES, 50 µM FeEDTA, MS microelements (Murashige and Skoog, 1962), 10 g l<sup>-1</sup> sucrose and 7 g l<sup>-1</sup> bacto-agar. Seedlings were grown on vertically placed Petri dishes in the growth chamber. Culture conditions were 21°C, with a 16 h day length at lighting levels of 120 µmol m<sup>-2</sup> sec<sup>-1</sup> photosynthetically available radiation (PAR), using neon tubes (in combination from Mazdafluor Blanc Industrie and Mazda fluor Prestiflux, Mazda Eclairage, France). For dark conditions, plates were wrapped in four layers of aluminium foil.

When plants were grown to maturity, seeds were either directly sown into soil in the greenhouse or plantlets were transferred from *in vitro* culture to soil. Greenhouse conditions were as follows: the photoperiod 16 h natural light with possible complementation (sodium lamp SON/T AGRO 400, Philipps, France) when light irradiance was lower than 220–260 µmol m<sup>-2</sup> sec<sup>-1</sup> and the thermoperiod was 23°/18°C (day/night). Irrigation was undertaken three times per week with nutrient solution (chemical fertilizer Solu-plant, 2 g l<sup>-1</sup>, Duclos International), and air humidity was regulated to approximately 55%.

### Mutant selection and genetic analysis

Approximately 500 M<sub>2</sub> lines generated following EMS mutagenesis were kindly provided by Prof. G. Belliard (Institut des Sciences Vegetales, Gif sur Yvette and University of Paris XI, Orsay, France). The EMS treatment (0.3% EMS, 16 h) resulted in the segregation of chlorophyll mutations in approximately 2–5% of the M<sub>2</sub> families. Fifty seeds from each individual M<sub>2</sub> family were sown on the medium described above supplemented with 0.1 µM 1-naphthaleneacetic acid (NAA).

Selected *sax1* plants were transferred to the greenhouse, self-fertilized and the transmission of the phenotype was confirmed in the M<sub>3</sub> generation. Plants from the M<sub>3</sub> generation were used as the female parent in crosses; back-crossing was repeated two successive times with wild-type plants from the Columbia ecotype. One hundred per cent of the F<sub>1</sub> plants resulting from these crosses (359 plants analyzed) exhibited a wild-type phenotype. The F<sub>2</sub> progenies of three populations were analyzed; the *sax1* phenotype segregated in a 1 (mutant): 3 (wild-type) ratio in agreement with the Chi-square statistical test (ratios were 128:367, 95:299 and 99:293). The F<sub>2</sub> segregating population of *sax1* generated after two back-crosses was further propagated to yield F<sub>4</sub> seeds which were used for all the experiments described.

For mapping, 33 seedlings homozygous for the *sax1* mutation were selected in an F<sub>2</sub> population issued from the cross of *sax1* (Col-0 background) and wild-type plants from the ecotype *Landsberg erecta* (Ler). Genomic DNA for PCR was prepared from leaves using the method described by Konieczny and Ausubel

(1993). Co-dominant cleaved amplified polymorphic sequence markers (CAPS) were used as described by Konieczny and Ausubel (1993) and simple sequence-length polymorphism markers (SSLP) as described by Bell and Ecker (1994). The CAPS and SSLP primer pairs were purchased from Research Genetics, Inc. (Huntsville, AL, USA). The map positions of the CAPS and SSLP markers were from the *Arabidopsis* map generated through the Landsberg/Columbia recombinant inbred lines by Lister and Dean (1993), released June 29 1995. The map distances relative to the CAPS and SSLP markers in centimorgans were calculated according to Kosambi (1944).

### Growth assays

Root growth inhibition by different hormones was carried out in two ways. Seedlings were germinated and grown either on hormone-supplemented media for 7 days or on plates containing minimal medium for 4 days and then transferred for an additional 2 days onto a minimal medium supplemented with various concentrations of a given hormone (root elongation on ABA).

Routinely, lengths of primary root of 30 seedlings were measured with a graduated ruler ( $\pm$  0.5 mm) under a binocular microscope. In transfer experiments, the elongated part of the root was measured by the same method 2 days after transfer. Standard errors calculated for the mean of 30 measurements per condition never exceeded 6% of the absolute value. Inhibition of elongation in the presence of an effector was calculated relative to elongation on minimal medium and expressed as a percentage for a comparison between wild-type and mutant genotypes.

To test the effect of 24-epibrassinolide (EBR), stimulation and/or inhibition of growth was assayed by measuring the length of the root and/or hypocotyl of wild-type and mutant 7-day-old seedlings germinated and grown directly in the presence of various concentrations of hormone (GA, ACC) or/and EBR. When ABA or IAA were tested together with EBR, 5-day-old seedlings grown on minimal medium were transferred onto the supplemented media for 2 days. The brassinosteroid EBR was solubilized in ethanol; the absence of ethanol effects on root and hypocotyl growth has been checked.

For whole mount light microscopy, 7-day-old seedlings grown in the light were stained with methyl-blue dye (0.001% (W/v) water solution. Determination of epidermal cell dimensions was performed directly under a microscope using an ocular micrometre (Leitz, Dialux 22). Measurements were performed on cells located in the middle part of the hypocotyl which is representative of the elongation of any epidermal cell throughout the entire growth phase of the hypocotyl (Gendreau *et al.*, 1997). In roots, epidermal cells were measured in the differentiation zone where cells are fully expanded.

### Germination and dormancy assays

For germination assays, mature seeds were plated on culture medium (see Growth conditions) supplemented with various ABA concentrations. After 4 days at 4°C in darkness to break seed dormancy, plates were transferred at 21°C upon a photoperiod 16 h light/8 h dark. Germination of seeds was scored as positive when the radicle tip had fully penetrated the seed coat. The percentage of germination was determined by dividing the number of seeds that germinated at a given time by the total number of plated seeds  $\times$  100.

For dormancy studies, plants of the wild-type and mutant genotypes were grown at the same time in the greenhouse.

Freshly harvested seeds were kept drying for 1 week before a chilling treatment for 0, 4, 8 and 10 days at 4°C in darkness. Germination of seeds was scored as described above.

#### Measurements of stomatal aperture

Plants in pots containing coarse sand were grown in a growth chamber. The temperature was 21°C during the light period (330  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ) and 19°C during the dark period; the relative humidity was constant at 70%. To study wild-type and mutant plants at a similar stage of development, mutant seeds were sown about 10 days earlier. Measurements of stomatal aperture were carried out on epidermal strips isolated from leaves of 5- to 6-week-old plants. Methods for epidermis peeling and measurements of stomatal aperture were derived from the ones applied to *Commelina communis* (Vavasseur *et al.*, 1995). For each treatment, 10 epidermal strips were floated on the incubation solution (60 mM KCl, 10 mM MES, pH 6.15), first in the absence of hormone for 2 h under light (450  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ), then in the presence of different ABA concentrations for 3 h.

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